Changes in phenotypic frequencies and the analysis of stress related traits of *Drosophila takahashii*: a study of seasonal acclimation

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Abstract: 【Aim】 Variation in coloration is frequently found in insects, but little is known about its functional value. *Drosophila takahashii* exhibits genetically coded discontinuous variation in abdominal melanisation. To determine whether physiological performance is likely to be affected by melanisation pattern, in this study we investigated the variation in abdominal melanisation and stress related traits among individuals belonging to three different color morphs, and tested the hypothesis that seasonal environmental conditions would enhance the adaptation of the corresponding seasonal phenotype. 【Methods】 *D. takahashii* flies were collected from altitudinal sites and a Mendelian analysis of genetic crosses from true breeding dark and light color strains confirmed the occurrence of a major locus, with dominance of D allele. Ecophysiological traits in populations as well as flies of three body color phenotypes were statistically analyzed. 【Results】 A significant increase in the frequency of the dark allele was observed during the dry season and lighter flies occur in the wet season, which suggests that climatic selection plays a role. However, intermediate flies were abundant in both seasons. There were significant F values for increase in all traits for intermediate phenotypes ($P < 0.001$) due to acclimation but no such acclimation effects were observed in dark and light true breeding strains of *D. takahashii* ($P \geqslant 0.42$). 【Conclusion】 As per our hypothesis, significantly higher physiological tolerance was observed in dark morph under cool-dry stress conditions, and in light morph under hot-wet conditions, respectively, as determined by different traits. Interestingly, intermediate phenotypes showed higher capability to acclimation to both conditions. Further, we found seasonal changes in temperature and humidity to confer selection pressures on stress-related traits.

Key words: *Drosophila takahashii*; allele frequency; life history variation; discrete phenotypes; heterozygote flexibility

1 INTRODUCTION

In ectotherms like insects, ecophysiological and behavioral traits contribute to their ecological success (Willmer *et al.*, 2005; Schowalter, 2006). Thermal adaptations are well known for insect taxa from different parts of the world (Lee and Denlinger, 1991; Hoffmann *et al.*, 2003), but effects due to other abiotic factors have received less attention. The tropics experience relatively consistent temperatures but there are significant seasonal variations in precipitation. Unlike temperature, there are few reports on the role of humidity as a selection agent for stress-related traits in various insect taxa (Danks, 2007). Tropical climatic conditions have an impact on wing pattern polyphenism in *Bicyclus* butterflies from Africa due to variation in temperature and humidity level (Roskam and Brakefield, 1999). Further, seasonally varying wet and dry forms in different species of butterflies show evolutionary responses due to differences in behavior, environment and nature of predation (Brakefield and Larsen, 1984; Brakefield and Reitsma, 1991). Thus, seasonally varying environments impose strong natural selection and cause rapid phenotypic changes in quantitative traits (Shapiro, 1976; Tauber and Tauber, 1981; Danks, 2007).

The evolutionary effects of thermal changes have been tested experimentally in different insects (Brakefield and Willmer, 1985; Berry and Willmer, 1986). Temperature extremes can have significant negative effects on physiological and life history traits of all organisms. Analysis of these deleterious effects is a major focus of attempts to understand the evolutionary consequences of environmental variation (Cossins and Bowler, 1987). Organisms can resist the deleterious effects of temperature extremes by evolving increased resistance to unfavorable conditions or by evolving

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acclimation abilities that allow facultative increases in resistance (Levins, 1969; Hoffmann and Parsons, 1991; Huey and Berrigan, 1996). The analysis of acclimation responses is important because acclimation can markedly alter stress resistance (Levins, 1969; Stanley *et al.*, 1980; Yamamoto and Ohba, 1984; Kimura, 1988; Watson and Hoffmann, 1996). Studies of acclimation in response to temperature have been hampered by a lack of genetic variation within populations (Hoffmann and Watson, 1993; Loeschcke *et al.*, 1994). In the present study, acclimation capacity of three body color phenotypes in *D. takahashii* was tested. If heterozygote phenotypes show increased variation in acclimation ability, they could be useful tools for exploring the genetic basis of acclimation responses and the costs of such responses.

Several studies showed beneficial effects of cold stress acclimation in diverse insect taxa but acclimation to desiccation stress received less attention (Bale, 2002; Hoffmann *et al.*, 2003). Enhanced drought tolerance was reported in a soil-dwelling springtail by pre-acclimation to a mild drought stress (Sjursen *et al.*, 2001), in *Folsomia candida* (Holmstrup *et al.*, 2002); in *Belgica antarctica* (Benoit *et al.*, 2007); and in *Cryptophgus antarcticus* (Elnitsky *et al.*, 2008). In contrast, a single study showed increased desiccation resistance in females of four *Drosophila* species due to dehydration acclimation (Hoffmann, 1991). Thermal fluctuations, ranging from sudden changes within days to gradual changes among seasons, impose selection pressures on organisms (Angilletta, 2009). In variable environments, selection favors genotypes that perform well over a wide range of temperatures (Levin, 1986; Gilchrist, 1995), resulting in tradeoffs between physiological performance and other aspects of the life history (Angilletta *et al.*, 2003). However, adaptive changes due to acclimation in diverse *Drosophila* species remain unknown so far.

Field observations and laboratory studies indicated differences in mating preferences of color morphs of two-spot ladybird beetle (Majerus, 1994). Laboratory selection experiments suggested increased or decreased level of mating preference in *Adalia bipunctata* (Majerus *et al.*, 1986). Further, seasonal differences in mating preference exist in *Harmonia axyridis*, *i. e.*, non-random matings between body color morphs occur during spring and summer (Osawa and Nishida, 1992). However, it is not known whether humidity changes could be a selective agent in the tropics. If there is rapid increase of the dark body color flies of *D. takahashii* during dry season, variations in climatic conditions such as temperature or humidity may affect mating preference between total body color variants in *D. takahashii*. Thus, short term exposure of high vs. low levels of thermal as well as humidity conditions on true breeding strains of body color morphs may show possible changes in mating preferences of *D. takahashii*. The effects of adult acclimation to cooler and drier (mimicking dry) versus hot and humid (mimicking wet season) on mating propensity of body color morphs of *D. takahashii* with no choice mating experiments were tested.

Species of the *takahashii*-subgroup occur from eastern Australia to Japan northwards and to the Indian subcontinent in the west, where *D. takahashii* is the most widespread species but remains unexplored for its adaptations to climatic stresses (Bock, 1980; Markow and O'Grady 2006). Seasonal changes in the frequencies of dark, intermediate and light morphs in *D. takahashii* populations along latitude were analyzed. Field data have shown seasonal variation in the frequency of dark and light morphs in *D. takahashii*, whereas numbers of heterozygote flies are abundant in both seasons. Seasonally varying wild-caught *D. takahashii* from five altitudinal localities (low- to mid-altitude) for stress related traits were investigated. Genetic basis of body color polymorphism was determined with genetic crosses between the true breeding dark and light strains of *D. takahashii*. Seasonally varying wild-caught body color phenotypes were tested for Hardy-Weinberg equilibrium to assess the effect of climatic selection. Populations for both the seasons were tested for geographical variation in various stress related traits. The true breeding strains for the dark and the light body color were tested for differences in stress related traits. The lack of thermal effects on body melanisation and ecophysiological traits in the laboratory populations grown at different developmental temperatures led us to compare fitness consequences of dark, intermediate and light phenotypes on exposure to seasonally mimicked cool, dry (dry) and hot-wet (wet) conditions. Current data suggest that changes in the frequencies of dark and light morphs are a consequence of seasonal variation in humidity during wet versus dry season. Present results support the role of varying humidity level in maintaining body color polymorphism and its impact on water balance in this tropical *drosophila* species.

2 MATERIALS AND METHODS

2.1 Cultures

Wild living *D. takahashii* individuals were collected during wet and dry seasons from five altitudinal sites (Chandigarh, 347 m; Kalka, 658 m; Gumman, 957 m; Koti, 1 126 m; Salogra, 1 520 m) of the western Himalayas. Wild-caught flies were used to initiate isofemale lines (30 – 40 per population) which were reared on cornmeal-yeast-agar medium at 21℃ and 60% RH. Climatic data for the sites of origin of populations were obtained from Indian Institute of Tropical Meteorology (IITM; www. tropmet. res. in).

Field experiments on mating success of *D. takahashii* flies were conducted at ten local sites (~1 – 2 km apart) at Gumman in wet (wet) as well as dry (dry) season.

2.2 Analysis of body color variation

Seasonally varying populations of *D. takahashii* were examined under Olympus stereozoom microscope SZ 61 (www. olympus. com, Japan) with Cell^{B} imaging software. Body color variations in flies captured from wild, flies having dark coloration or no coloration over all the abdominal segments were observed. Three body color phenotypes (dark, intermediate and light) in females of *D. takahashii* were recognized. Melanisation was estimated from dorsal view of the abdomen giving values ranging from 0 (no pigment) to 10 (complete darkness) for each abdominal segment in wild-caught individuals of *D. takahashii*. Since the abdominal segments differ in size, relative sizes were multiplied with segment wise pigmentation scores (Parkash *et al.*, 2008). The data on percent melanisation were calculated as ($\sum$ observed weighted melanisation scores of six abdominal segments per fly/$\sum$ relative size of each segment $\times$ 10 per fly) $\times$ 100 (Parkash *et al.*, 2008). For stress related traits, flies were analyzed soon after getting into the laboratory during each season.

2.3 Genetic basis of body color variation

For establishing homozygous strains for dark and light morphs of *D. takahashii*, three approaches were followed. (a) Isofemale lines were established from the wild caught females (60 – 80) of each population and their progeny were checked for 8 successive generations. No homozygous lines for light or dark morph were obtained and in all the lines segregating light and dark flies were found. (b) Virgin flies from laboratory cultures were isolated and made several single pair matings (40 – 50). The crosses gave all light or dark progeny, which were taken as homozygous strains. However, only 1 – 2 homozygous strains were obtained for light and dark morphs, respectively, through this method. (c) 30 – 40 mated pairs were aspirated from fallen fruits in the gardens in wet as well as dry seasons. The progeny of each mated pair was screened for homozygous light and dark strains. This method provided 5 light strains from flies collected during wet season; and 3 dark strains from dry season collection. In order to ascertain the genetic basis as well as dominance of the allele, Mendelian crosses (F_1 and F_2 crosses) were carried out with these homozygous light and dark strains of *D. takahashii*.

2.4 Thermal and humidity laboratory selection

For selection experiments, fifteen isofemale lines from one midland (Gumman, 957 m) were used. Each isofemale line was allowed to lay eggs at 21℃; 60% RH in multiple replicate food vials. Two replicates with eggs for each isofemale lines were transferred to different humidity and thermal conditions. For each isofemale line, two replicates were maintained at 25℃ and high humidity (80% RH); two at 25℃ and low humidity (40% RH); two at 17℃ and high humidity and two at 17℃ and low humidity. The isofemale lines were maintained under these conditions continuously for 10 generations. Subsequently, on 11th generation, the effect of selection temperature and humidity on various ecophysiological traits (desiccation resistance, rate of water loss, heat knockdown, cold stress and mating success) were measured on flies reared at both different thermal and humidity conditions.

2.5 Desiccation resistance assay

To measure desiccation resistance, ten laboratory reared flies per isofemale line per population were isolated in a dry plastic vial, which contained 2 gm of silica gel at the bottom and were covered with a disc of foam piece. Such vials with foam plugs were placed in a desiccation chamber (Secador electronic desiccator cabinet) which maintains 4% – 5% relative humidity. The desiccation survival data were also obtained for 10 true breeding strains (10 replicates each) of the dark as well as the light body color. The vials were inspected every hour and the number of dead flies (completely immobile) was recorded. The survival curves as a function of desiccation hours at 21℃ were drawn for the dark, intermediate and the light phenotypes.

2.6 Analysis of rate of water loss

For calculation of the rate of water loss in dark, intermediate and lighter strains of *D. takahashii*, method of Wharton (1985) was followed, modified by Benoit *et al.* (2010) and Yoder *et al.* (2009). Total body water content (m) was calculated as the

difference between wet or fresh (f) and dry mass (d), *i. e.*, $m = f - d$. Individual flies were weighed and placed at 0.00 a_v (a_v = percent RH/100) for a specified time at one hour intervals (1 – 8 h), and reweighed. The rate of water loss was derived from the slope of regression line on a plot of $\ln(m_t/m_0)$ against time according to Wharton's equation (Wharton, 1985) and modified by Benoit *et al.* (2010):

$$m_t = m_0 \, e^{-k_t}.$$

Where m_t is the water lost at time t, and m_0 is the initial water content. Rate (k_t) is the slope of regression line and expressed as percent per hour.

2.7 Assessment of cuticular lipid mass

Cuticular lipid mass was estimated individually on eight-day old flies (15 I. F lines × 10 replicates) of each body color phenotype by following Gibbs method (Gibbs *et al.*, 2003). Flies were dried overnight at 60℃ to get dry mass, *i. e.*, devoid of body water. Each dried fly was kept in HPLC-grade hexane in 2 mL eppendorf tube (www. tarson. com) for 1 hour and thereafter it was removed from the solvent and was again dried at room temperature and finally reweighed on a sartorius microbalance (Model-CPA26P; with precision 0.001 mg; www. sartorious. com). Cuticular lipid mass per cm^2 was calculated as the difference in mass following solute extraction divided by surface area (cm^2). There were significant F values for increase in all traits for intermediate phenotypes ($P < 0.001$) due to acclimation but no such acclimation effects were observed in dark and light true breeding strains of *D. takahashii* ($P \geqslant 0.42$). The surface area was calculated by following Edney's formula $12M^{0.67}$ (Edney, 1977).

2.8 Trehalose estimation

For trehalose estimation, 10 flies of each isofemale line (15 IF lines × 10 replicates each) were homogenized in a homogenizer (Labsonic® M; www. sartorious. com) with 300 μL Na_2CO_3 and incubated at 95℃ for 2 hours to denature proteins. An aqueous solution of 150 μL acetic acid (1 mol/L) and 600 μL sodium acetate (0.2 mol/L) was mixed with the homogenate. Thereafter, the homogenate was centrifuged (Thermo-Scientific, Fresco 21; USA) at 12 000 r/min for 10 minutes. The supernatant of this homogenate was used for estimation of trehalose.

For trehalose estimation, aliquots (200 μL) were placed in two different tubes; one was taken as a blank while the other was digested with trehalase at 37℃ (Megazyme trehalose assay kit; K-Treh 10/10; www. megazyme. com). In this assay, released D-glucose was phosphorylated by hexokinase and ATP to glucose-6-phosphate and ADP which was further coupled with glucose-6-phosphate dehydrogenase and resulted in the reduction of nicotinamide adenine dinucleotide (NAD). The absorbance by NADH was measured at 340 nm (UV-2450-VIS; USA). Pre-existing glucose level in the sample was determined in a control reaction lacking trehalase and was subtracted from total glucose concentration.

2.9 Thermotolerance assays

For thermoresistance traits, flies were individually isolated in separate vials after one minute anesthesia with di-ethyl ether followed by one day recovery period. Thermoresistance assays were made individually on 150 flies per population (10 flies × 15 isofemale lines). There were significant F values for increase in all traits for intermediate phenotypes ($P < 0.001$) due to acclimation but no such acclimation effects were observed in dark and light true breeding strains of *D. takahashii* ($P \geqslant 0.42$). Further, for dark, intermediate and light body color strains, the present study analyzed ten flies from each of the ten strains (10 flies × 10 strains) for cold mortality assay. Effects due to age, sex, anesthesia, ambient room temperature and thermal conditions of assay vials were controlled.

For heat knockdown assay, individual flies were placed in 5 mL glass vials submerged into a water bath at a constant temperature of 37℃. Flies were scored for time (in minutes) taken to be knocked down. Heat mortality was scored as percent of dead flies under variable time duration for heat stress at 37℃ for 5 – 35 minutes at five minute interval.

For cold mortality as a function of duration of stress, thirty groups of ten flies either for dark or light or intermediate phenotypes were transferred without anesthesia in empty 5 mL glass vials. These vials were set in thermocol boxes (24 cm × 13 cm × 10 cm) containing ice flakes (made with an ice flaking machine AICIL) which were kept at 0℃ in the refrigerator. Batches of three vials were removed after four hour interval, *i. e.*, from 4 to 36 hours, for trait analysis. This was followed by transfer of flies to petri-plates (9 cm diameter) in a temperature controlled room at 25℃ and percent mortality was recorded. For population analysis, chill-coma recovery period was recorded after 8 hours of cold stress (at 0℃) on 150 flies per population.

2.10 Assessment of acclimation responses

To measure the acclimation pretreatment time duration for desiccation resistance, 10 female individuals of each replicate (15 I. F lines × 10

replicates each) were subjected to desiccation stress at ~0 – 5% relative humidity. The initial body water content in replicate groups was recorded. The time period in which flies lost ~15% – 17% body water was assessed as pre-treatment time duration. Further, for recovery period, individuals were placed on non-nutritive agar and tested at hourly intervals for increase in body water till the lost body weight was regained. Acclimated flies were subjected to desiccation stress until death in order to test the increased desiccation resistance due to acclimation. Increased desiccation survival hours were calculated after subtracting the desiccation resistance hours of non-acclimated (control) from acclimated individuals. Control and treatment experiments were run simultaneously under identical experimental conditions. Rate of water loss in control and acclimated flies was estimated simultaneously following Wharton's method. Further, to study the effect of acclimation on trehalose content, desiccation acclimated flies were subjected to trehalose estimation.

Darker, intermediate and lighter body color strains of *D. takahashii* reared at 21℃ were acclimated to cold (14 ± 0.1℃) temperatures for chill coma study. For testing responses to high temperature acclimation, flies grown at 21℃ were subjected to higher (25℃) temperatures. For both groups, adults were acclimated for 6 days and were checked for chill coma recovery and heat knockdown, respectively.

2.11 Analysis of mating propensity

For mating propensity experiments, laboratory reared virgin females and males of dark and light morphs were subjected to hot-wet (25℃ and 80% RH) and cool-dry (15℃ and 40% RH) conditions. Control experiments (21℃ and 60% RH) were also conducted. In each mating chamber, 5 virgin females and 5 virgin males of each dark morph as well as of light morph were placed and observations on 10 such pairs were made for 60 minutes under no-choice conditions. For all the observed matings, percent mated pairs (MP), mating success, mating latency (ML) and copulation period (CP) were recorded. In this way, matings were observed for 10 true breeding strains (in 10 replicates) for all the experiments.

2.12 Statistical analysis

For analyses of ecophysiological traits in populations as well as flies of three body color phenotypes, mean trait values ± *S. E.* or *S. D.* were used for tabular data and illustrations. For Mendelian crosses, χ^2 test was applied to find fit between observed and expected number of dark, intermediate and light phenotypes (Zar, 1996). For each population, data on body color phenotypes of wild-caught flies for each season were tested for Hardy-Weinberg Equilibrium. Data on desiccation hours (LT_{50} or LT_{100}) of the flies of three body color phenotypes were compared with ANOVA. Nested ANOVA was used for data on desiccation related traits under varying thermal and humidity conditions. Percent data were arcsine transformed and then subjected to ANOVA. In order to test whether matings under no-choice experiments were random or assortative, contingency χ^2 tests were performed for control as well as different stress experiments. Further, increase in stress resistance and trehalose content in acclimated flies as compared to non-acclimated (control) were analyzed through ANOVA. Statistica software was used for calculations and illustrations (Statsoft Inc., Release 5.0, Tulsa, OK, USA).

3 RESULTS

3.1 Genetic basis of body color variation

A comparison of wild-caught *D. takahashii* collected during two different seasons showed differences in total body color phenotypes, *i. e.*, flies collected in the two different seasons exhibited three distinct body color phenotypes (dark, intermediate and light, Fig. 1: A). Flies with intermediate body color (melanisation = ~30% – 34%) phenotype were distinct from either dark (melanisation = 59%) or light (melanisation = ~6%) phenotypes and were more in number as compared with darker and lighter phenotypes in both the seasons (Fig. 1: A). No overlapping of phenotypes was found during the scanning of wild-caught samples of *D. takahashii*. There were no quantitative differences in melanisation score. However, melanisation scores of flies on a continuous scale showed three discrete classes, *i. e.*, light flies showed 7.43% ± 1.05% melanisation score, while dark and intermediate color flies exhibited 58.21% ± 1.32% and 30.05% ± 2.22% melanisation, respectively. These results support the occurrence of discontinuous variations in total body melanisation of *D. takahashii*.

Genetic crosses with true breeding strains (n = 5 for the dark and the light body color strains, respectively) showed intermediate phenotype in the F_1 progeny; and 1∶2∶1 (dark, intermediate and light) in F_2 progeny and the data on such genetic crosses are given in Table 1. Chi-square analyses have shown non-significant differences between observed vs. expected number of dark, light and

intermediate body color phenotypes (Table 1). There was not any overlapping of body color phenotype in the F_2 progeny. These results were consistent with a single gene model for total body color variation in *D. takahashii*. Thus, the dark and light body color morphs may represent diallelic variation at an autosomal locus. There was incomplete dominance because F_1 flies were intermediate in body color.

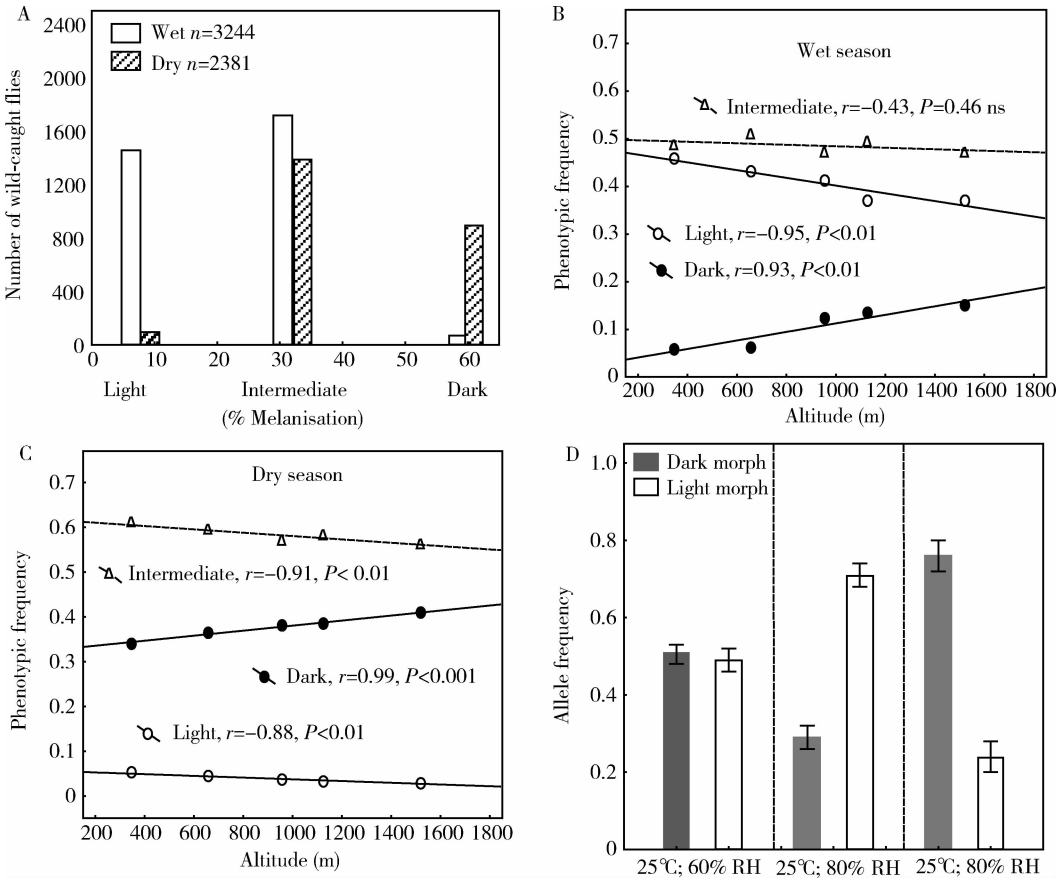

Fig. 1 Percent melanisation of total body color for wild-caught *Drosophila takahashii* flies of rainy (wet) and autumn (dry) season from Gumman (957 m) showing discrete differences as three body color phenotypes (dark, intermediate and light) (A), changes in the frequencies of dark, intermediate and light body color phenotypes as a function of changes in altitude of sites of origin of *D. takahashii* populations (B – C), and changes in allele frequencies under varying thermal and humidity conditions (D).

Table 1 Data on genetic crosses (F_1 and F_2) between true breeding strains for dark (D) and light (L) body color in *Drosophila takahashii*

Genetic crosses	Type	Replicate no.	n	Segregating phenotypes			χ^2	*P*-value
				D	I	L		
(A) Light ♀ × Dark ♂	F_{1A}		305	–	305	–	–	–
		1	165	40	81	44	0.05	0.82
		2	203	52	103	48	0.04	0.83
F_{1A} × F_{1A}	F_2	3	189	50	90	49	0.43	0.51
		4	177	43	93	41	0.46	0.49
		5	153	38	74	41	0.16	0.68
(B) Dark ♀ × Light ♂	F_{1B}		262	–	262	–	–	–
		1	201	52	100	49	0.005	0.94
		2	188	47	90	51	0.33	0.56
F_{1B} × F_{1B}	F_2	3	196	46	99	51	0.02	0.87
		4	156	35	83	38	0.64	0.42
		5	149	38	77	34	0.17	0.67

n: Total number of flies scored; ns: Non-significant; I: Intermediate. F_{1A} and F_{1B} represents reciprocal crosses. χ^2 test was used to test differences between observed and expected number of phenotypes.

3.2 Seasonal changes in body color phenotypes

Data on phenotypic frequencies of three body color phenotypes for wild-caught *D. takahashii* collected during wet and dry seasons from five altitudinal localities are given in Table 2. During seasonal changes, the frequencies of darker and lighter body color phenotypes are quite different, *i.e.*, light phenotype (wet: 0.37 – 0.46; dry: 0.029 – 0.051; Fig. 1: B – C), dark (wet: 0.05 – 0.15; dry: 0.37 – 0.46; Fig. 1: B – C) but intermediate phenotype is more abundant during both seasons (0.47 – 0.60; Fig. 1: B – C). There is significant increase in the frequency of dark morph from wet to dry season (0.05 – 0.46). Seasonal data on field populations of *D. takahashii* showed deviations from Hardy-Weinberg equilibrium (HWE) for all the populations collected during season changes and chi-square analyses have shown significant differences between observed vs. expected number of dark, light and intermediate body color phenotypes (Table 2).

Table 2 Data on phenotypic frequencies of three body color phenotypes (dark, intermediate and light) for wild caught flies of *Drosophila takahashii* collected during wet and dry seasons from six altitudinal localities

Population (Altitude in meters)		Wet season						Dry season					
		Body color phenotypes				χ^2 for HWE	*P*-level	Body color phenotypes				χ^2 for HWE	*P*-level
		n	D	I	L			n	D	I	L		
Chandigarh (347)	O	919	54	444	421	20.56	***	578	199	350	29	61.33	***
	E		83	387	449				242	264	72		
Kalka (658)	O	657	40	334	283	20.78	***	540	197	320	23	56.16	***
	E		66	284	307				236	242	62		
Gumman (957)	O	771	94	361	316	34.33	***	447	183	248	16	54.14	***
	E		98	353	320				211	192	44		
Koti (1 126)	O	615	62	339	214	18.65	***	366	145	208	13	34.39	***
	E		87	289	239				169	159	38		
Salogra (1 520)	O	415	48	233	134	12.46	***	483	204	265	14	40.56	***
	E		66	198	151				234	206	43		

*** $P < 0.001$; D: Dark; I: Intermediate; L: Light; n: Number of wild caught *D. takahashii* female flies for each population and season; χ^2 values and *P*-level for Hardy-Weinberg equilibrium (HWE) are also given.

3.3 Stress tolerance of the body color morphs

Desiccation survival curves for dark and light morphs are shown in Fig. 2 (A). Dark morph showed ~2 fold higher values for LT_{50} as well as LT_{100} for desiccation survival as compared with light morph. Comparisons at LT_{50} ($t_{1,9} = 42.36$, $P < 0.001$) as well as at LT_{100} ($t_{1,9} = 49.78$, $P < 0.001$) are significant for desiccation resistance between dark and light morph. Intermediate phenotypes have values in-between the darker and lighter body color phenotypes, these values were non-overlapping and significantly different. There was significant difference in the rate of water loss of the three phenotypes (1.8%/hour in darker and 2.6%/hour in intermediate versus 3.2%/hour in lighter phenotype of *D. takahashii*) (Fig. 2: B). The dark and the light morph for mortality after short term exposure to cold stress were compared and the results are shown in Fig. 2 (C). All the three phenotypes showed significant differences in their mortality under cold stress (dark morph = 37%; intermediate = 64% and light morph = 100% mortality corresponding with 20 hours treatment under cold stress). By contrast, under heat stress the trend for mortality reverses, *i.e.*, dark morph = 100%; intermediate = 50% and light morph = 10% mortality corresponding with 40 minutes treatment under heat stress at 37℃ (Fig. 2: D).

3.4 Assessment of acclimation effects on body color phenotypes

There was lack of acclimation effect in dark and light phenotypes ($P = 0.38$) but a significant increase in desiccation resistance in intermediate phenotype ($P < 0.001$) when acclimated to short-term desiccation stress (Fig. 3: A, B). Desiccation resistance is higher in intermediate adults after acclimation (control: 17.33 h; acclimated: 26.80 h). For intermediate phenotype, trehalose content increased significantly in acclimated flies. Results suggest that increased trehalose content and decreased rate of water loss after adult acclimation might be associated with increased desiccation survival hours. Further, intermediate flies also responded to cold (Fig. 3: C) and heat (Fig. 3: D). Table 4 illustrates the comparison of desiccation resistance, rate of water loss, trehalose content, heat knockdown and chill coma resistance (mean ± *SE*; 5 lines × 10 replicates for each phenotype) in non-acclimated (control) and acclimated female flies of *D. takahashii*. For all these physiological traits, there was significant increase in trait value in acclimated intermediate flies, but such responses were not observed in true breeding darker and lighter flies of *D. takahashii*. Further, results of ANOVA

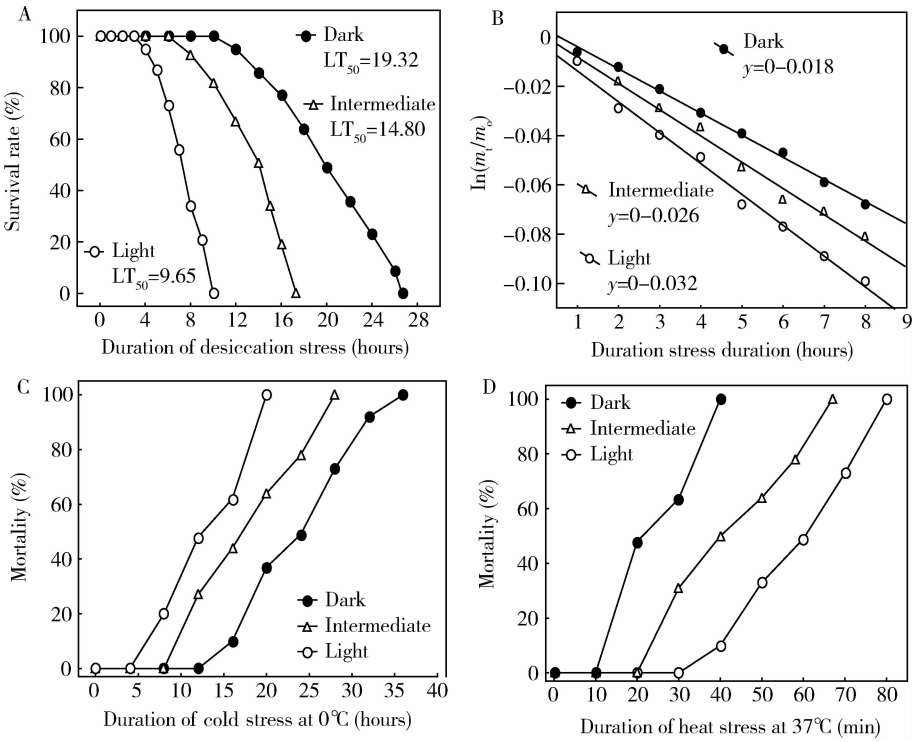

Fig. 2 A comparison of desiccation survival curves (A), rate of water loss according to Wharton's method (B), percent mortality due to cold stress (C) and heat stress (D) of flies with three body color phenotypes of *Drosophila takahashii*

Changes in trait values are shown as a function of different duration of stress treatments. Data are based on 10 flies × 10 replicates for (A); individual flies × 30 replicates of each body color phenotype (B – D).

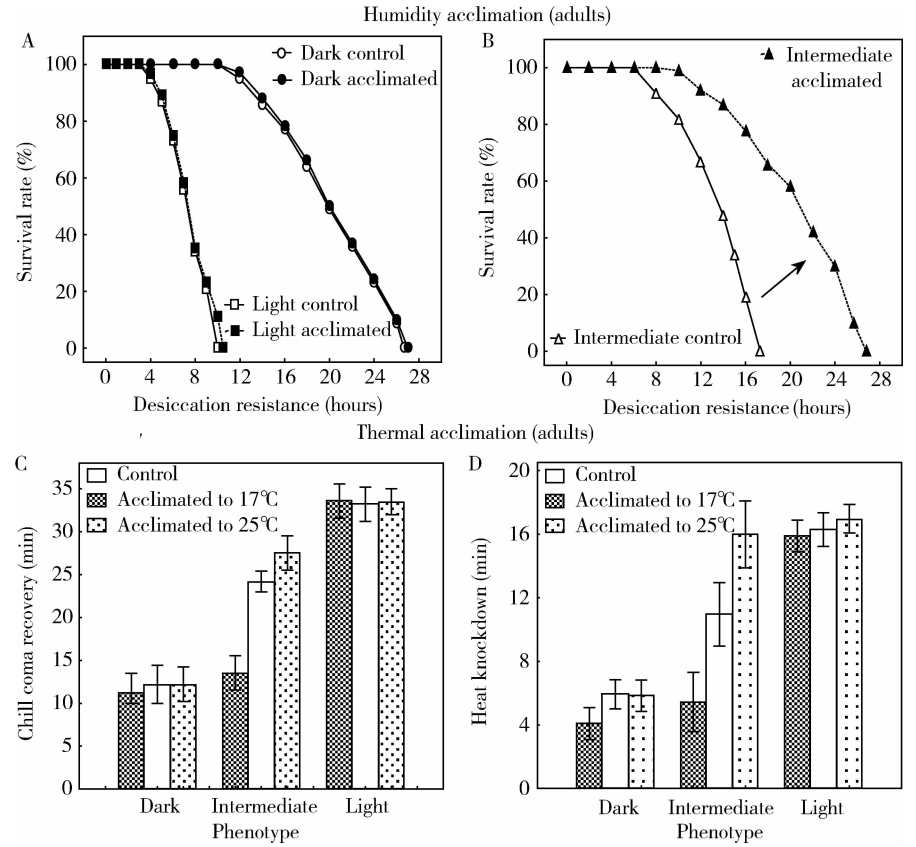

Fig. 3 A comparison of stress resistance in control and acclimated flies (adults) of three body color phenotypes of *Drosophila takahashii*

Desiccation survival curves of dark and light phenotypes (A) and of flies with intermediate phenotype (B), chill coma recovery (C) and heat knockdown (D) of adult flies of three body color phenotypes acclimated to 17 and 25℃. Bars represent mean ± *SE* (10 individuals × 10 replicates).

Table 3 Data (mean ± *SE*) for changes in desiccation resistance, rate of water loss, trehalose content, heat and cold resistance in acclimated flies in dark, intermediate and light phenotypes as compared with control flies of *Drosophila takahashii*

Phenotype		Desiccation (h)	RWL (μg/h)	Trehalose content (μg/fly)	Heat knockdown	Chill coma recovery
1. Dark	Control	26.66 ± 1.21	0.018 ± 0.003	53.21 ± 2.11	5.29 ± 1.10	12.21 ± 2.01
	Acclimated	27.00 ± 1.13	0.018 ± 0.002	53.50 ± 1.98	5.48 ± 1.36	11.25 ± 1.58
ANOVA	*MS*	21.23	0.012	14.32	26.21	123.21
	F-value	4.84 ns	0.58 ns	4.23 ns	1.22 ns	3.01 ns
2. Intermediate	Control	17.33 ± 1.30	0.026 ± 0.002	37.12 ± 1.65	10.05 ± 0.98	24.21 ± 1.25
	Acclimated	26.80 ± 0.98	0.019 ± 0.003	50.56 ± 2.21	15.95 ± 1.00	13.56 ± 2.22
ANOVA	*MS*	2 510.83	0.045	2 132.14	541.44	1 105.21
	F-value	3 230.11 ***	1 723.21 ***	4 317.56 ***	1 558.82 ***	1 462.25 ***
3. Light	Control	10.50 ± 1.21	0.032 ± 0.003	26.21 ± 1.25	16.29 ± 2.01	33.21 ± 1.89
	Acclimated	10.30 ± 0.58	0.032 ± 0.001	26.58 ± 1.87	17.94 ± 1.25	33.59 ± 2.17
ANOVA	*MS*	13.21	0.034	7.57	9.98	11.23
	F-value	1.21 ns	0.89 ns	1.14 ns	2.36 ns	2.14 ns

RWL: Rate of water loss; *MS*: Mean square; ns: Not significant; *** $P < 0.001$. Results of ANOVA testing the effects of adult acclimation to desiccation stress, heat or cold stress for the female flies of three body color phenotypes of *D. takahashii*.

explaining the trait variability are shown in Table 3. There were significant *F* values for increase in all traits for intermediate phenotypes ($P < 0.001$) due to acclimation but no such acclimation effects were observed in dark and light true breeding strains of *D. takahashii* ($P \geqslant 0.42$).

4 DISCUSSION

D. takahashii, is the only widespread species of *takahashii*-species subgroup (Bock, 1980; Markow and O'Grady, 2006). However, adaptations of *D. takahashii* to climatic stresses remain unknown so far. The study investigated *D. takahashii* flies from wet vs. dry seasons for desiccation related traits. Interestingly, desiccation survival hours of flies from dry season are about two fold higher than flies of wet season. The mechanistic basis of such differences in water conservation under drier dry season is linked with total body color polymorphism in this tropical species. There is a rapid increase in the frequency of the dark flies during dry season while the light body color flies are more pronounced during wet season. Body color variations in *D. takahashii* are consistent with genetic polymorphism generating alternative phenotypes with different levels of environmental stress tolerances. Thus, seasonally varying selection pressures favor alternative phenotypes in different seasons and thereby maintaining genetic polymorphism.

Changes in abiotic environments exert strong selection pressure on the water balance mechanisms of terrestrial insect taxa including drosophilids (Chown and Nicolson, 2004). Under drier habitats, total body water loss occurs through three routes: via excretion, respiration and cuticular transpiration. Excretory and respiratory losses are quite low (< 15 percent) and cuticular transpiration accounts for > 80 percent of total water loss in most insect species (Gibbs, 2002). Thus, cuticular transpiration contributes a major part of total body water loss in insects. Mechanisms underlying the evolution of desiccation resistance differ between various insect taxa, *i. e.*, reduction in cuticular transpiration may occur either through cuticular lipids (Hadley, 1994; Parkash *et al.*, 2008) or body melanisation (Parkash *et al.*, 2008). Reduction in body water loss due to changes in the amount of cuticular lipids was found in several insect taxa such as scorpions, beetles and ants (Toolson and Hadley, 1977; Hadley *et al.*, 1981; Zachariassen *et al.*, 1987). However, studies using *Drosophila* species did not find a relationship between water loss and surface lipids (Gibbs *et al.*, 1997; Gibbs, 1998, 2002). Besides lipids, melanin is another hydrophobic cuticular component but its effects on cuticular transpiration were not considered by earlier studies on water balance in *Drosophila* species (Rajpurohit *et al.*, 2008). Some studies showed the relationship between melanisation and total body water loss in *Drosophila* species (Parkash *et al.*, 2008; Rajpurohit *et al.*, 2008). Thus, changes in body melanisation can affect total body water loss in *Drosophila* species (Parkash *et al.*, 2010).

4.1 Genetic polymorphism for body color

Ectothermic insects from tropics experience significant seasonal variations in precipitation. Seasonally varying wet and dry forms occur in different tropical species of butterflies (Brakefield and Larsen, 1984; Brakefield, 1987; Brakefield and Reitsma, 1991). Further, genetic polymorphism for body color has been reported in different insect taxa (Majerus, 1998). However, genetic polymorphism for body color is known for few *Drosophila* species. So far, a single study has reported total body color dimorphism (black and

brown morphs distributed allopatrically in South-East Asia) under the control of a single locus in *D. elegans* but the ecological significance of such genetic polymorphism is not clear (Hirai and Kimura, 1997). For *D. takahashii*, body color variations and other ecophysiological traits remained unexplored. However, in the present study, seasonal populations of *D. takahashii* helped us to characterize genetic polymorphism for body color phenotypes. The light body color morph is desiccation sensitive with high rate of cuticular water loss and this corresponds with its distribution in humid tropics and during wet season in subtropics. Due to higher desiccation resistance and reduced rate of cuticular water loss in dark morph, this species has adapted to drier habitats in the dry season. Melanisation in intermediate phenotype fluctuate between seasons, and correspondingly the ecophysiological traits.

4.2 Ecological significance of body color polymorphism: analysis of seasonal changes

In the present study, genetic polymorphism for total body color variation and its impact on desiccation related traits, *i. e.*, there are adaptive differences between dark, intermediate and light morphs for various ecophysiological traits, was observed. Firstly, there was a rapid increase in morph frequency under seasonally varying climatic conditions in the field (Fig. 1; Table 2). Secondly, higher cold tolerance of the dark morph over the light morph is also evident from measures of cold mortality as a function of duration of cold stress (100 percent for light phenotype, 64 percent for intermediate and 37 percent for dark phenotype after 20 hour cold stress). There was a trade-off in the energy metabolites, *i. e.*, higher trehalose content in dry flies confers longer survival under desiccation stress. In contrast, wet flies have low amount of trehalose content, and hence low levels of desiccation resistance. Finally, for desiccation resistance, there was lack of changes in the amount of cuticular lipids between the dark, intermediate and light body color phenotype. Thus, cuticular lipids cannot account for differences in desiccation resistance and rate of body water loss in the dark, intermediate vs. light body color morph of *D. takahashii*. A major conclusion is that dark morph is positively correlated with resistance to cold and desiccation stress. In contrast, the light followed by intermediate phenotypes confers adaptive responses under hot and humid conditions. Thus, significant differences for various ecophysiological traits in dark, intermediate and light phenotypes of *D. takahashii* are in agreement with their seasonal adaptations.

4.3 Effect due to adult acclimation

In *Drosophila* species, acclimation responses to desiccation related traits were examined under different thermal conditions (Gibbs *et al.*, 1998). In contrast, the changes due to habitat specific humidity levels on desiccation acclimation have been less documented (Hoffmann, 2010). It has been argued that ability to show acclimation response might be associated with varying thermal and humidity levels in natural conditions (Hoffmann, 1991) and cyclic changes in humidity levels were associated with desiccation related traits in wild populations of *Drosophila* species (McKenzei and Parsons, 1974; Parkash *et al.*, 2010). In the present study, the effect of low humidity acclimation in desiccation related traits in different phenotypes of *D. takahashii* was tested. Homozygote dark and light phenotypes showed lack of acclimation response for desiccation resistance (Table 3). However, dark and light phenotypes differ significantly in desiccation resistance and rate of water loss. Current results showed rapid increase in desiccation resistance and trehalose level in intermediate flies (Table 3) acclimated to low humidity conditions. The differences in resistance between dark and light is consistent with their ecological distribution, dark individuals are adapted to cool, dry conditions whereas light phenotypes can be found in locales where temperature is high and conditions are humid. In this study, desiccation acclimation ability has also been associated with species distribution patterns, *e. g.*, *D. birchii*, a rain forest *Drosophila* species lacks acclimation response while species with widespread distribution have shown acclimation effects. Results in the present study have shown increase in desiccation resistance as well as thermo-tolerance in intermediate phenotype due to acclimation but there is lack of effects in homozygote dark and light flies and such changes are consistent with their abundance level under field conditions.

For *D. takahashii*, occurrence of body color polymorphism and its ecological significance has not been reported previously. True breeding strains at 21℃ for the dark and the light body color morphs of *D. takahashii* were isolated and data on genetic crosses are consistent with single gene model and incomplete dominance between dark and light body color alleles. Body color phenotypes of wild-caught flies (dark, intermediate and light) showed deviations from Hardy-Weinberg equilibrium during wet as well as dry seasons. In wild, the intermediate phenotypes remained in excess in both seasons.

Thus, seasonal changes in frequency of body color phenotypes in *D. takahashii* represent genetic responses to selection pressure imposed by climatic conditions. Dark, intermediate and light body color phenotypes significantly differ in resistance to desiccation and cold. The abundance of light morph during wet season matches its adaptations to humid habitats while dark morph is better adapted to drier habitats during the dry season. Thus, dark/light color dimorphism is an adaptive mechanism of *D. takahashii*. Further, there is a need to find out the advantage of excessive heterozygotes found in nature as well as under true breeding crosses of *D. takahashii*. This is the first report to show the genetic basis of body color polymorphism, seasonal variations in the frequencies of body color phenotypes, and morph differences in ecophysiological traits (resistance to desiccation or cold or heat) and acclimation capacity of dark, intermediate and light body color phenotypes in *D. takahashii*.

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高桥氏果蝇表型频率变化和胁迫相关性状的分析：季节性适应研究

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摘要:【目的】昆虫中经常可见体色变化,但是关于其作用价值了解甚少。高桥氏果蝇 *Drosophila takahashii* 在遗传上表现出腹部黑化的不连续变化。为了测定生理性状是否可能受黑化的影响,我们调查了 3 个不同色型个体腹部黑化的变化和胁迫相关性状,检验了季节性环境条件将增强相应的季节性表型的适应性这一假说。【方法】从不同海拔地点采集高桥氏果蝇,对深色和浅色纯育品系的遗传杂交进行的孟德尔分析证实存在一个主要位点,为 D 等位基因显性。对种群以及 3 种色型果蝇的生理生态学性状进行了统计分析。【结果】在旱季观察到深色等位基因频率的显著增加,在雨季会出现体色较浅的果蝇,这说明气候选择起着重要作用。不过,在这两个季节中体色居中的果蝇均很多。中间色型的果蝇由于适应而表现出所有性状的 F 值均显著增加($P<0.001$),但在深色和浅色纯育品系中未观察到这类适应效果($P\geq0.42$)。【结论】通过不同性状的测定结果我们提出,在干冷胁迫条件下,在深色型中观察到显著更高的生理学耐性;而在湿热条件下,在浅色型中观察到显著更高的生理学耐性。有意思的是,中间表型在这两种条件下均有较强的适应能力。而且,我们发现温湿度的季节性改变给胁迫相关的性状施加了选择压力。

关键词: 高桥氏果蝇; 等位基因频率; 生活史变化;分离的表型; 杂合体的灵活性

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